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A CAPILLARY ELECTROPHORESIS-TANDEM MASS SPECTROMETRY METHODOLOGY FOR THE DETERMINATION OF NON-PROTEIN AMINO ACIDS IN VEGETABLE OILS AS NOVEL MARKERS FOR THE DETECTION OF ADULTERATIONS IN OLIVE OILS.

LAURA SÁNCHEZ-HERNÁNDEZ, MARIA LUISA MARINA, ANTONIO L. CREGO *

Department of Analytical Chemistry, Faculty of Chemistry, University of Alcalá. Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

*Corresponding author (phone + 34-918855076; fax + 34-91-8854971; e-mail antonio.crego@uah.es)

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Abstract

A new analytical methodology based on capillary electrophoresis-mass spectrometry (CE-MS^2) is presented in this work, enabling the identification and determination of six non-protein amino acids (ornithine, β-alanine, GABA, alloisoleucine, citrulline and pyroglutamic acid) in vegetable oils. This methodology is based on a previous derivatization with butanol and subsequent separation using acidic conditions followed by on-line coupling to an ion trap analyzer for MS^2 detection established through an electrospray-coaxial sheath flow interface. The electrophoretic and interface parameters were optimized obtaining the separation of all compounds in less than 15 min and with resolutions higher than 5. The proposed method was validated by assessing its accuracy, precision (RSD < 7% for corrected peak areas), LODs and LOQs (between 0.04-0.19 ng/g and 0.06-0.31 ng/g, respectively) and linearity range (R^2 □ 0.99), and it was used in order to identify the selected non-protein amino acids in soybean oils, sunflower oils, corn oils and extra virgin olive oils. MS^2 experiments performed the fingerprint fragmentation of these compounds allowing to corroborate ornithine and alloisoleucine in seed oils but not in olive oils. The method was applied to identify and quantify olive oil adulterations with soybean oil detecting in a single run the amino acids in mixtures up to 2% (w/w). The results showed a high potential in using these compounds as novel markers for the detection of adulterations of extra virgin olive oils with seed oils. Thus, the developed method could be considered a simple, rapid and reliable method for the quality evaluation of extra virgin olive oil permitting its authentication.

1. Introduction

Non-protein amino acids are naturally occurring amino acids of low molecular weight which are not usually found as protein constituents. The number of known non-protein amino acids is quite large, more than 800, and their origins are diverse as are their functions [1]. Most of them have been isolated from plants, foods, fungi, and microorganisms and they are usually considered as secondary compounds [2]. The nutritional and agricultural importance of non-protein amino acids lies in the fact that many occur in plants grown for food and fodder and can have beneficial as well as toxic properties. Thus, some of these compounds, such as ornithine and homoserine, are recognized as intermediates or end products in the synthesis of protein amino acids or in primary metabolism in both plants and animals [2,3], other can play roles as defensive agents, as antibacterial or antifungal, or as drugs for the treatment in human diseases (e.g. L-DOPA) [4-6] while some of them can present toxic properties (e.g. mimosine) [7-9].

The importance of olive oil and in particular the extra virgin olive oil has been correlated with its benefits in human health [10-12]. A relevant aspect of oil authenticity is adulteration of extra virgin olive oils with lower price oils which is prohibited by European regulations [13]. Thus, the genuineness of olive oils is a very important aspect from the point of view of quality and marketing.

To establish their authenticity, a wide number of chromatographic (as HPLC, GC, HPLC-MS, GC-MS, or CE) [14] or spectrometric techniques (as fluorescence, NMR, Raman, Infrared, MS or chemiluminiscence) followed by multivariate statistical analysis of the data obtained [15] have been described.

Chromatographic methods are based on the determination of particular chemical compounds in oils. Their coupling with MS supposes powerful techniques that provide resolving power and structural information to unequivocal confirmation of the presence of particular species. In the literature, coupling techniques such as GC-MS [16-18] and HPLC-MS

[19-23] have been described for the authentication of olive oils blended with soybean, sunflower or corn oil, being the saponifiable fraction (98.5-99.5% of the oil) the most studied part. Thus, by GC-MS, fatty acids [16,17] and volatile compounds [18] have been described as markers. In these methods, adulterations with seed oils up to 1% (w/w) previous the derivatization of the fatty acids to methyl esters were detected [17] and up to 0.01% (w/w) was identified following two chemometric approaches to carefully interpret the wide accumulated data obtained (566 samples) and to detect subtle differences among them [14]. On the other hand, using HPLC-MS, triacylglycerols [19,20], the alcoholic fraction [21], glycerophospholipids [22] and sterols [23] have been reported. However, only the works using triacylglycerols as markers demonstrated the possibilities of the developed methods for detecting mixtures of olive oils with seed oils up to 1% (w/w) followed by a multivariate method [19].

Although the presence of amino acids as minor components in vegetable oils has been described by direct infusion MS [24] and HPLC-UV [25] methods, the presence of non-protein amino acids in vegetable oils has not been reported.

The aims of this work were the following: (i) to develop a sensitive method based on capillary electrophoresis with MS detection (CE-MS) enabling the simultaneous determination of six non-protein amino acids in vegetable oils, (ii) to apply the developed method to investigate their presence in oils, and (iii) to propose these compounds as novel markers for the detection of adulterations in olive oils.

2. Experimental

2.1 Chemicals

All reagents employed were of analytical grade. Methanol, chloroform and isopropanol were supplied from Scharlau Chemie (Barcelona, Spain). Sodium hydroxide, hydrochloric acid

and 25% (v/v) ammonium hydroxide solution were supplied from Merck (Darmstadt, Germany). Formic acid was from Riedel-de Häen (Seelze, Germany). γ-Aminobutyric acid (GABA) and β-alanine were supplied from Sigma (St. Louis, MO, USA). Pyroglutamic acid, alloisoleucine, ornithine, citrulline and hydrogen chloride/1-butanol solution were from Fluka (Buchs, Switzerland). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Standards and samples

The stock standard solutions of non-protein amino acids were prepared from a 1 mg/mL solution in acetonitrile/water (40:60, v/v) and diluting them to the desired concentration. These solutions were stored at room temperature before use. 500 µL of the standard solutions were evaporated at 80 ºC and 15 mbar in an eppendorf tube before to their derivatization.

Arbequina, Picual, and Hojiblanca extra virgin olive oils, refined sunflower oils, refined corn oils and refined soybean oils were acquired in different supermarkets (Madrid, Spain) from different trademarks. See Table 1 for more information.

The sample preparation was carried out considering a previous method [26]. Briefly, 40 g of vegetable oils and extra virgin olive oils were weighed and extracted with 160 mL of methanol:chloroform (2:1, v/v) and left at -20ºC overnight. Then, their centrifugation (4000g, 15 min, 4 ºC) was carried out. The upper phase was collected in a new tube and, when the bottom phase was washed with 100 mL of methanol/chloroform/water (2:1:0.8, v/v/v), the new upper phase was combined. The mixed fractions were then washed with 40 mL of chloroform and 100 mL of water, centrifuged (4000g, 15 min, 4 ºC) and the aqueous phase was separated for its evaporation to dryness in a concentrator at 80 ºC. Finally, the derivatization with butanol was carried out before injection in the CE system.

2.3 Derivatization procedure

In MS methods, the butylation of amino acids containing mono- and dicarboxylic acid groups greatly improves ionization efficiencies and hence analytical sensitivity [27]. Moreover, esterification improves the mass differentiation among the analytes increasing the selectivity.

The butyl ester derivatization was adapted from a previous procedure [28]. Thus, butanol derivatizing agent was added to the evaporated extract of the samples (0.5 mL to standards and 1 mL to real samples) and shaken in a vortex. Then, the reaction was carried out in an oven at 80 ºC during 30 min. After 5 min in freezer at -4ºC to stop the reaction, the derivatization agent excess was evaporated in a concentrator at 80 ºC to dryness. Finally, reconstitution of analytes was achieved in 500 µl of acetonitrile/water (40:60, v/v).

2.4 Capillary Electrophoresis-Mass Spectrometry conditions

The analyses were carried out in a $HP^{3D}CE$ instrument (Agilent Technologies, Palo Alto, CA, USA) coupled through an orthogonal electrospray interface (ESI, model G1607A from Agilent Technologies, Palo Alto, CA, USA) to an 3D Ion Trap mass spectrometer (model 1100 from Agilent Technologies, Palo Alto, CA, USA) for MS detection. LC/MSD Trap Software 5.2 was used for MS control and data analysis. Uncoated fused-silica capillaries of 50 µm id with 60 cm of length were used. Before first use, a new capillary was conditioned by rinsing at 1 bar with 1 M NaOH for 20 min, then with water for 5 min and 0.1 M HCl for 5 min and finally with buffer (0.1 M formic acid pH = 2.0) for 30 min. After each run, the capillary was rinsed at 1 bar for 2 min with buffer. Injections were made at the anodic end using a N_2 pressure of 50 mbar for 50 s. The electrophoretic separation was achieved with a voltage of 25 kV (positive polarity). The temperature of the capillary was kept constant at 25 °C.

MS operating conditions were optimized by adjusting the needle-counter electrode distance, composition and flow rate of sheath liquid, and nebulizer and drying gas conditions.

Electrical contact at the electrospray needle tip was established using a sheath liquid based on isopropanol:water (50:50, v:v) with 0.1 % formic acid and delivered at a flow rate of 3.3 µL/min by a syringe pump (model 100, Holliston, USA) with SGE syringe of 10 mL from Supelco (Bellefonte, PA, USA). The nebulizer and drying gas conditions were 2 psi N_2 and 3 L/min N_2 at 300 ºC. The mass spectrometer operated with the ESI source in the positive ion mode (4.5 kV) and the m/z range scanned was from 50 to 280. The ion trap parameters were programmed in "smart mode" (software mode that provides automatic optimization of some ion trap parameters for precursor ions) with 50 % of compound stability and 100 % of trap drive level. Finally, the ion charge control mode operated to accumulate 100000 ions, for a maximum accumulation time of 300 ms with one scan. The fragmentation was carried out by collision induced dissociation with the helium present in the trap for 10 ms with fragmentation amplitude of 1.00 V, and isolation width of 4.0 m/z to obtain MS^2 spectra during the run in Multiple Reaction Monitoring (MRM) mode. Extracted ion electropherograms (EIEs) were obtained using a smoothed option of the software (Gauss at 1 point).

3. Results and discussion

3.1 Derivatization Optimization

Previous to the introduction of the amino acids in the CE-MS system, the percentage of the derivatization reaction was investigated for two aromatic protein amino acids, phenylalanine and tryptophan, using UV detection. When the derivatization into butyl esters of evaporated phenylalanine (0.151 µmol) and tryptophan (0.122 µmol) was achieved with 500 µL derivatizing agent (5.87 mmol) at 60 ºC and for 20 min as derivatization time, four peaks appeared corresponding to derivatized and underivatized amino acids. A percentage for the derivatization reaction about 95% was obtained for each compound. In order to improve the efficiency of the derivatization reaction, the

influence of the temperature and the derivatization time were investigated from 60 to 100 ºC ~~(60, 70, 80, and 100ºC)~~ and from 10 to 30 min ~~(10, 20, 30 min)~~, respectively. As can be seen in Table 2, from values of 80 ºC and 30 min the percentage of the derivatization reaction was 100% being these conditions chosen as optimal to achieve the derivatization of the amino acids.

3.2 Identification of non-protein amino acids

Six non-protein amino acids: citrulline, ornithine, pyroglutamic acid, β-alanine, GABA and alloisoleucine were chosen for their determination in vegetable oils because they have been identified in sunflower seeds (β-alanine, alloisoleucine, GABA, pyroglutamic acid) [29], soybean seeds (β-alanine, GABA, ornithine, citrulline) [30,31] and/or corn seeds (GABA, β-alanine, ornithine and pyroglutamic acid) [32].

The first experiments were carried out individually in order to identify the spectra of the different derivatized amino acids in MS and MS^2 modes. The selected buffer was formic acid 0.1 M (pH 2.0) due to the fact that all the amino acids presented positive charge at acidic pH. All derivatized amino acids were identified, although for pyroglutamic acid a non-expected quasi-molecular ion was produced during the derivatization (m/z 260). Fig. 1 shows the proposed structures of the six derivatized amino acids. Note that due to the formation of the butyl derivative of the amino acids, the quasi-molecular ions have sizes ($\geq$ 150 m/z) where the MS background noise is usually lower [33]. Once confirmed the quasi-molecular ions in MS mode, MS^2 experiments were performed. Fig. 1 illustrates the MS^2 spectra obtained for the six non-protein amino acids and the fragmentations of each molecule. These fragmentations were studied by MS^n experiments to elucidate the fragmentation pattern of the precursor ions. Thus, neutral losses of m/z 56 corresponding to the derivatizing agent ($CH_3CH_2CH=CH_2$) were produced for β-alanine, alloisoleucine, ornithine and GABA. Additional losses of water,

ammonia and or CO_2 molecules were also generated. For citrulline only the rupture of ammonia generated a stable compound of m/z 215. Finally, for pyroglutamic acid, the MS^n experiments also allowed to identify the quasi-molecular ion. Thus, MS^3 spectra using as precursor ions m/z 260 (in MS^1) and m/z 242, 186, 158 and 130 (in MS^2) concluded that pyroglutamic acid was derivatized with a double butyl chain and its quasi-molecular ion at m/z 260 contained a water molecule which was not completely desolvated during the ionization. Figure 2 shows the complex fragmentation pattern for pyroglutamic acid. As it can be seen, from m/z 242 two neutral losses of m/z 56 resulted in the product ions of m/z 186 and 130, respectively. The product ion 158 was obtained by a neutral loss of m/z 102 from m/z 260. Finally, the product ion m/z 84 was obtained by the decarboxylation from ion m/z 130 and by a neutral loss of m/z 56 and a water molecule from ion m/z 158.

3.3 Optimization of Capillary Electrophoresis-Mass Spectrometry conditions.

The separation conditions were optimized with mixtures of the six standard non-protein amino acids in order to obtain the maximum signal to noise (S/N) ratio and resolution between compounds. The first experiments in MS mode showed the following order of elution of the derivatized compounds: ornithine, β-alanine, GABA, alloisoleucine, citrulline and pyroglutamic acid. When a capillary of 60 cm was used (minimum possible length for the CE-MS equipment), resolutions higher than 5 between peaks were achieved in about 13 min as it can be seen in Fig. 3A.

On the other hand, MS^2 experiments in MRM mode were used to improve the selectivity and sensitivity and to carry out the identification of all betaines in one run. The ion trap parameters were optimized by separation of the compounds in the CE-MS system by MRM mode in order to achieve at least 10 points per peak and to obtain good sensitivity in S/N and precision. Thus, values for the maximum accumulation time from 50 to 300 ms, number of

scans averaged from 1 to 3 and the fragmentation time between 10 and 40 ms were studied. With lower values of maximum accumulation time and scans averaged, the points per peaks increased, however, the S/N decreased and the precision got worse. The chosen optimal conditions were 300 ms, one scan and fragmentation time of 10 ms to obtain at least 12 points per peak and RSDs of S/N lower than 10%.

Moreover, in order to increase the sensitivity for real samples, after the derivatization, the samples were reconstituted in acetonitrile/water (40:60, v/v) to carry out a stacking sample preconcentration according to our previous work [26]. Thus, a hydrodynamic injection of 50 s was employed. Fig. 3B illustrates the MS^2 electropherograms for the compounds in MRM mode under the optimal conditions.

3.4 Analytical characteristics of the method

The method was validated in terms of selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision and accuracy.

There are two reasons for considering the selectivity of the method developed as excellent. The first one is that it enables to reach a resolution among the amino acids exceeding base-line separation (see Fig. 3). The second is that according to the EC Decision about analytical methods operation and results interpretation [34], the unequivocal analysis of the compounds was possible monitoring at least two precursor-product ion transitions using MS^2 experiments, except in the case of citrulline, where only one transition was achieved.

LODs and LOQs for the amino acids were defined, respectively, as the minimum analyte concentration yielding a S/N ratio equal to 6 and 10 times [35]. According to this definition, if a concentration is equal to 0.04 ng/g of ornithine, β-alanine, GABA and alloisoleucine, or 0.19 ng/g of citrulline and pyroglutamic acid, they can be detected with only 5% chance of

committing a false positive or false negative. Table 3 shows the values for these limits obtained for the solutions previous to their derivatization.

The linearity was established using the external standard calibration method from six standard solutions containing the amino acids from their LOQ to 100 times the LOQ. The solutions were injected in triplicate and checked for linearity during three days. Calibration curves were established by considering the corrected peak areas (Ac, peak area to migration time ratio) from the EIEs. Satisfactory results were obtained in terms of linearity with a correlation coefficient higher than 0.99 for the average calibration plot, and with all the confidence intervals at 95% for intercept, including the 0 value (see Table 3).

The precision study was carried out for a real sample of a seed oil containing the amino acids as a sunflower oil (RSO-1) and was evaluated in terms of instrumental repeatability, method repeatability and intermediate precision (see Table 3). The calculated values of relative standard deviations (RSDs in %) for corrected peak areas, without including citrulline which was not detected in the samples, were lower than 3% and 7.0% for instrumental repeatability and intermediate precision, respectively.

The accuracy of the method was evaluated as the recovery obtained for each amino acid when a representative extra virgin olive oil (HEVOO-1) was spiked with 0.125 ng/g and 12.5 ng/g of each amino acid. The samples were injected in triplicate. Values of recovery ranging from 83% to 91% with RSDs $\leq$ 4% were obtained (see Table 3).

3.5 Determination of non-protein amino acids in vegetable oils

The method was applied to the determination of the six non-protein amino acids in real samples. Three different samples of each kind of seed oils and nine different samples of three different varieties of extra virgin olive oil were analyzed. The quantification of the samples was carried out by single-point standard addition calibration since the presence of matrix

interferences in vegetable oils was demonstrated previously by CE-UV [26]. Thus, it was performed by the injection of two different sample solutions for each vegetable oil sample: the sample solution and the spiked sample containing a known amount of amino acids (0.1 µg/mL of citrulline, GABA and alloisoleucine, and 1 µg/mL of ornithine, β-alanine and pyroglutamic acid). Table 4 shows the results obtained in the analyzed samples. Ornithine, β-alanine, GABA, alloisoleucine and pyroglutamic acid were determined for the first time in soybean, corn and sunflower oils while citrulline was not detected in these oils. The highest content was obtained for pyroglutamic acid and the lowest for GABA, being this about 10 times lower. For extra virgin olive oils, β-alanine, GABA and pyroglutamic acid were determined although with contents around 7, 5 and 12 times lower than in seed oils, respectively. These results are in agreement with the literature confirming the traceability of these non-protein amino acids from sunflower, soybean and corn seeds to their corresponding oils, except in the case of citrulline detected in soybean seeds but not in oils.

Finally, the usefulness of the developed method was evaluated by analyzing mixtures of olive oil samples with seed oil as soybean oil. Thus, percentages of 2, 5 and 10 % (w/w) corroborated the presence of ornithine and alloisoleucine in the olive oil mixtures (see Table 4). Fig. 4 shows the EIEs in MS^2 obtained for both compounds in a soybean oil sample (RSYO-3), an extra virgin olive oil sample (HEVOO-1) and, the oil mixture of HEVOO-1 with a 5% and 2% (w/w) of RSYO-3. Moreover, their unequivocal identification through their MS^2 spectra in the oil mixtures was confirmed. Thus, ornithine and alloisoleucine are proposed as novel markers for the detection of adulterations of olive oils with other vegetable oils such as soybean, corn and sunflower oils. In addition, concentrations higher than 0.5 ng/g of β-alanine, 0.2 ng/g of GABA or 1.0 ng/g of pyroglutamic acid in olive oils would suppose their adulteration with other vegetable oils. All of this confirms the high potential of the developed method to easily assess the quality of olive oils.

4 Concluding remarks

A very sensitive method CE–ESI-MS^2 for the simultaneous determination of six non-protein amino acids in vegetable oil samples was developed. Adequate analysis time (less than 15 min) and acceptable precision of the developed method were obtained (RSD < 7%). Excellent LODs were obtained, between 0.04 and 0.19 ng/g. Ornithine, β-alanine, GABA, alloisoleucine and pyroglutamic acid were determined in soybean, corn and sunflower oils while citrulline was not detected in these oils. β-Alanine, GABA and pyroglutamic acid were determined in extra virgin olive oils while ornithine, alloisoleucine and citrulline were not detected. Thus, ornithine and alloisoleucine are proposed as novel markers for the detection of adulterations of olive oils with seed oils such as soybean, corn and sunflower oils. The developed method is the first one in which the possibilities of CE-MS to investigate olive oil adulterations have been demonstrated and shows a high potential to easily assess the quality of olive oils.

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Figure Captions

Fig. 1. MS^2 spectra and structure of precursor ions for each non-protein amino acid are shown. CE conditions: BGE, 0.1 M formic buffer (pH 2.0); uncoated fused-silica capillary, 50 µm ID×85 cm; injection by pressure at 50 mbar×15 s; applied voltage, 25 kV; temperature, 25 ºC. ESI conditions: positive ion mode; spray voltage, 4.5 kV; sheath liquid, isopropanol/water (50/50 v/v) with 0.1% formic acid at 3.3 µL/min; drying gas flow, 3 L/min; drying temperature, 300 ºC; nebulizer pressure, 2 psi. Ion trap conditions: maximum accumulation time, 300 ms; averages, 3; scan, 50-350 m/z; MS^2 transitions with width, 4 m/z; fragmentation amplitude, 1.00 V; fragmentation time, 40 ms.

Fig. 2. MS^2 fragmentation pattern of pyroglutamic acid.

Fig. 3. **A)** CE-MS base peak electropherogram for standard amino acids mixture of 5 µg/mL each one (injection by pressure at 50 mbar×15 s). **B)** Simultaneous CE-MS^2 EIE for a standard amino acids mixture of 5 µg/mL each one (injection by pressure at 50 mbar×50 s). CE Conditions: uncoated fused-silica capillary, 50 µm ID×60 cm; Other CE conditions and ESI conditions as in Fig. 1. Ion trap conditions: maximum accumulation time, 300 ms; averages, 1; scan, 50-280 m/z. MS^2 transitions in MRM mode with width, 4 m/z; fragmentation amplitude, 1.00 and fragmentation time, 10 ms. Peak 1. Ornithine, 2. β-Alanine, 3. GABA, 4. Alloisoleucine, 5. Citrulline and 6. Pyroglutamic acid.

Fig. 4. CE-MS^2 EIE for ornithine **(A)** and alloisoleucine **(B)** in a) soybean oil sample (RSYO-3), b) extra virgin olive oil sample (HEVOO-1), c) oil mixture of HEVOO-1 with a 5% (w/w) of RSYO-3 to ornithine and with a 2% (w/w) of RSYO-3 to alloisoleucine, d) MS^2 spectra for ornithine and alloisoleucine in the oil mixture, respectively. All other experimental conditions were as in Fig. 3.

Fig. 1.

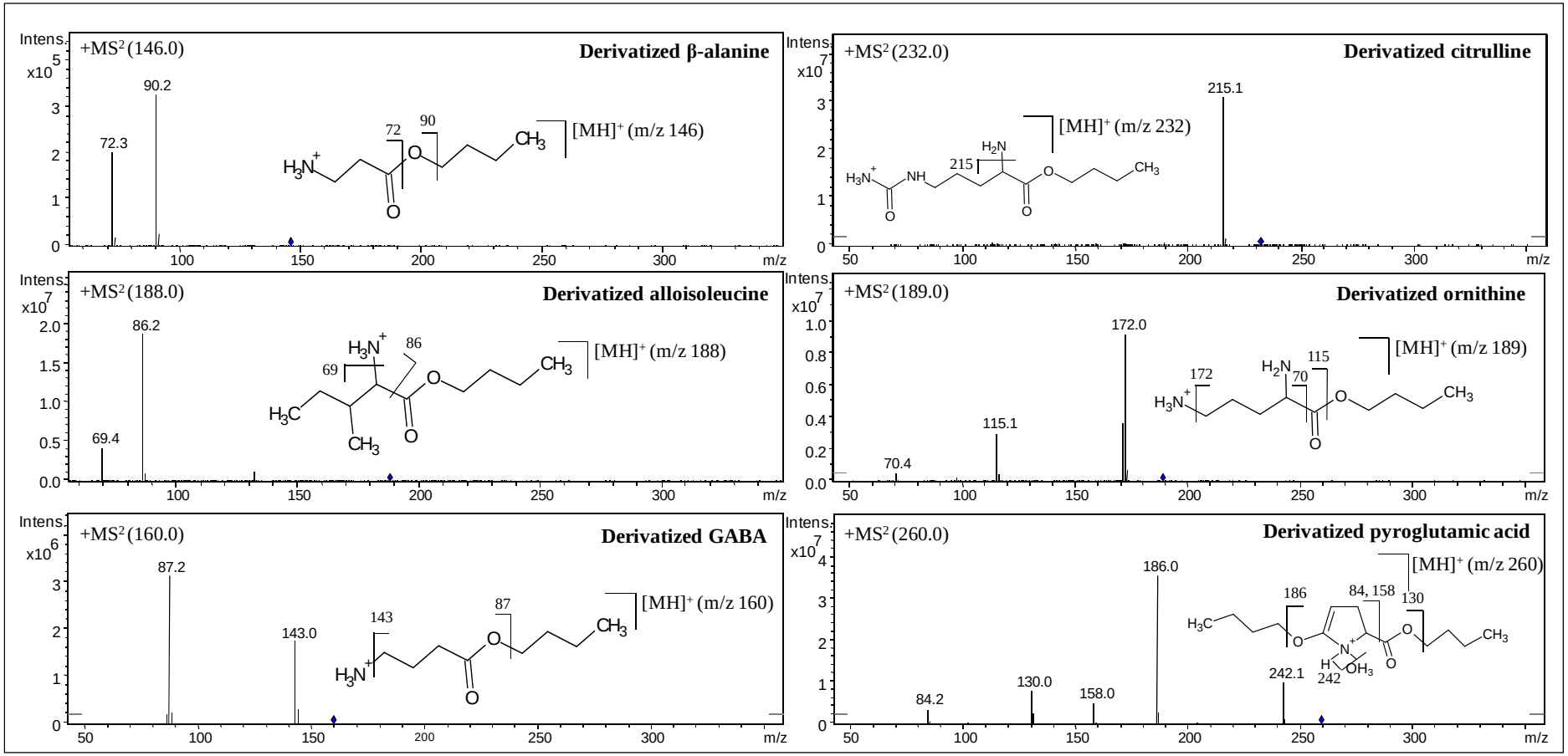
Derivatized β-alanine
+MS²(146.0)
[MH]⁺ (m/z 146)
Derivatized citrulline
+MS²(232.0)
[MH]⁺ (m/z 232)
Derivatized alloisoleucine
+MS²(188.0)
[MH]⁺ (m/z 188)
Derivatized ornithine
+MS²(189.0)
[MH]⁺ (m/z 189)
Derivatized GABA
+MS²(160.0)
[MH]⁺ (m/z 160)
Derivatized pyroglutamic acid
+MS²(260.0)
[MH]⁺ (m/z 260)
Intens.
m/z

Fig. 2.

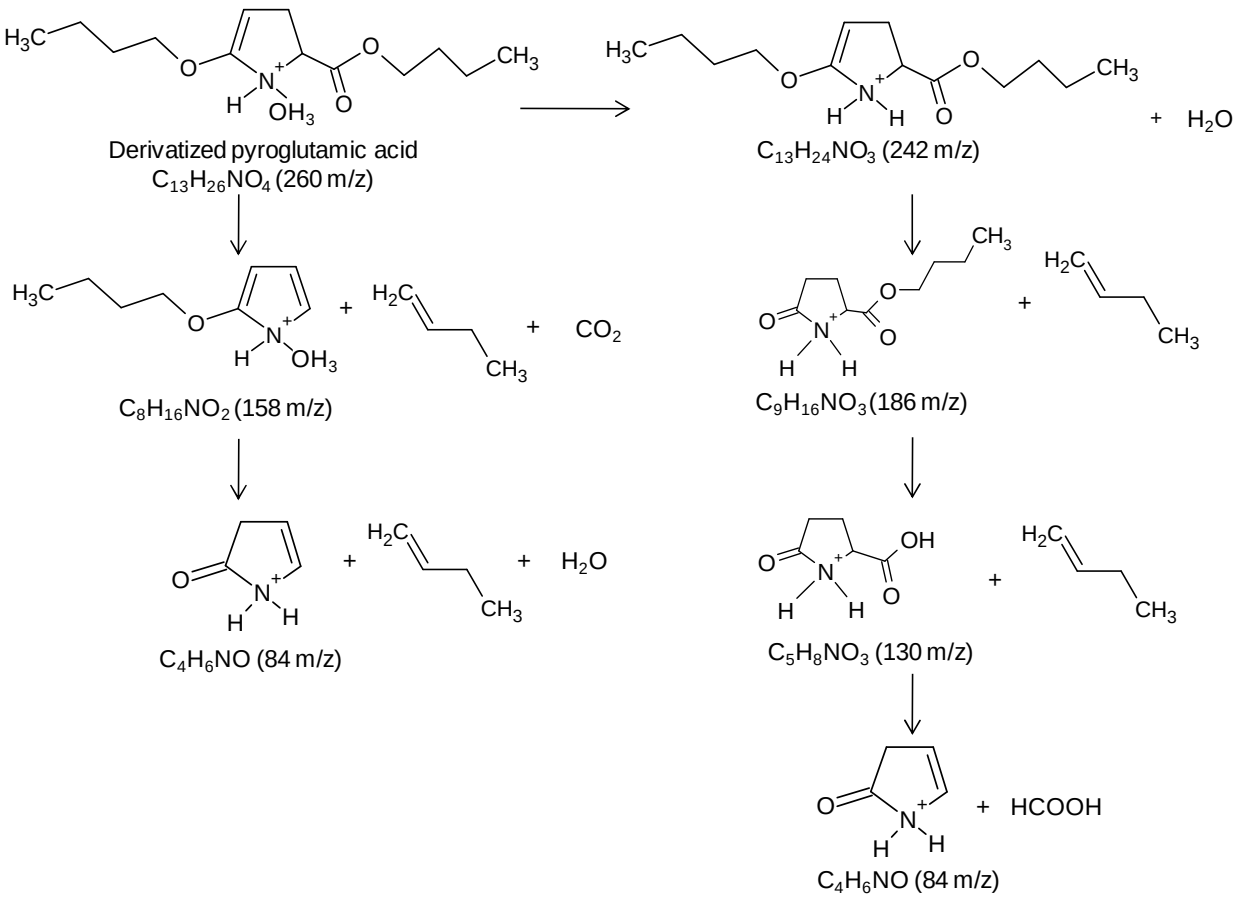
Derivatized pyroglutamic acid
C13H26NO4 (260 m/z)
C13H24NO3 (242 m/z)
+ H2O
C8H16NO2 (158 m/z)
+ CO2
C9H16NO3 (186 m/z)
C4H6NO (84 m/z)
+ H2O
C5H8NO3 (130 m/z)
+ HCOOH
C4H6NO (84 m/z)

Fig. 3.

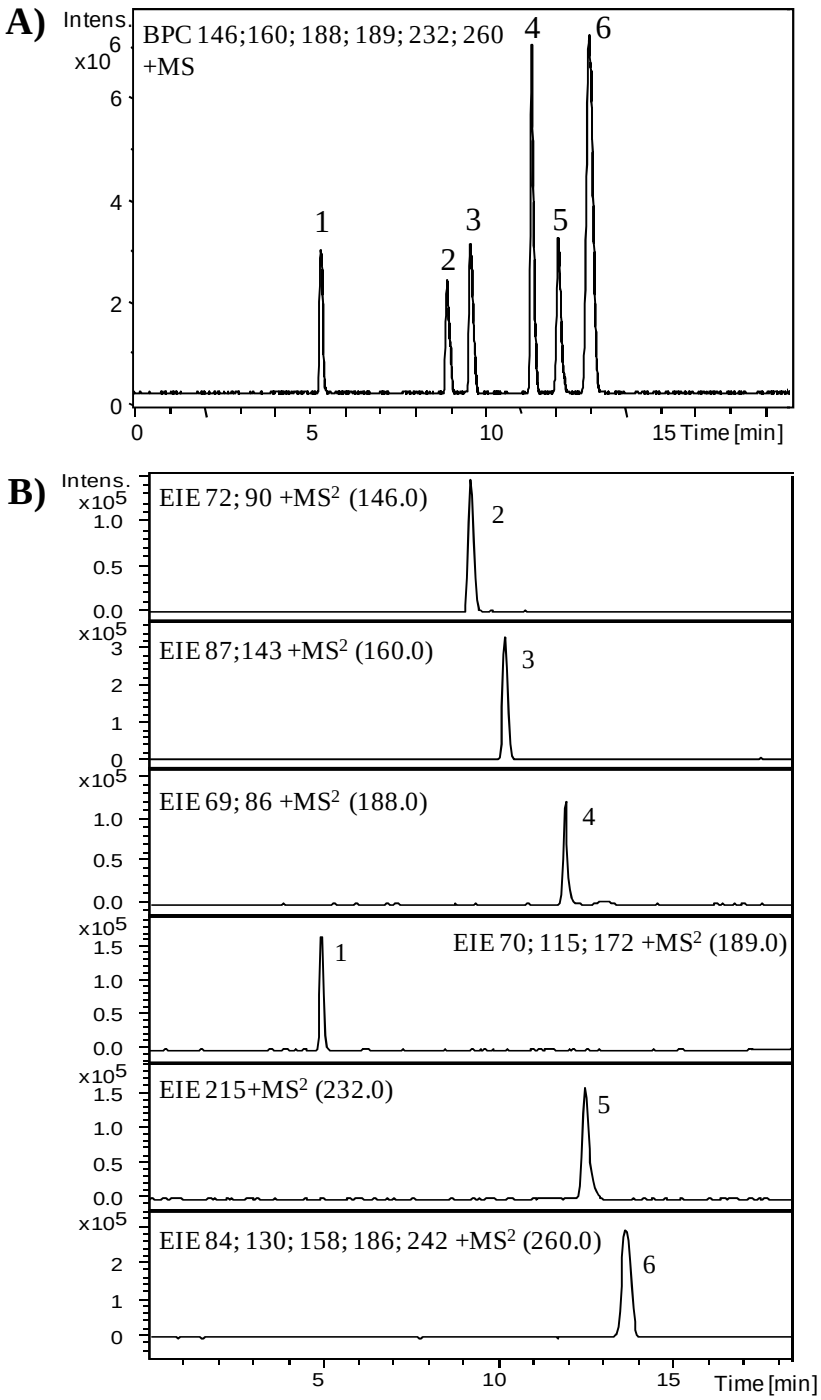
A)
Intens.
x10^6
BPC 146;160; 188; 189; 232; 260 +MS
1
2
3
4
5
6
Time [min]
B)
Intens.
EIE 72; 90 +MS2 (146.0)
EIE 87;143 +MS2 (160.0)
EIE 69; 86 +MS2 (188.0)
EIE 70; 115; 172 +MS2 (189.0)
EIE 215+MS2 (232.0)
EIE 84; 130; 158; 186; 242 +MS2 (260.0)
Time [min]

Fig. 4.

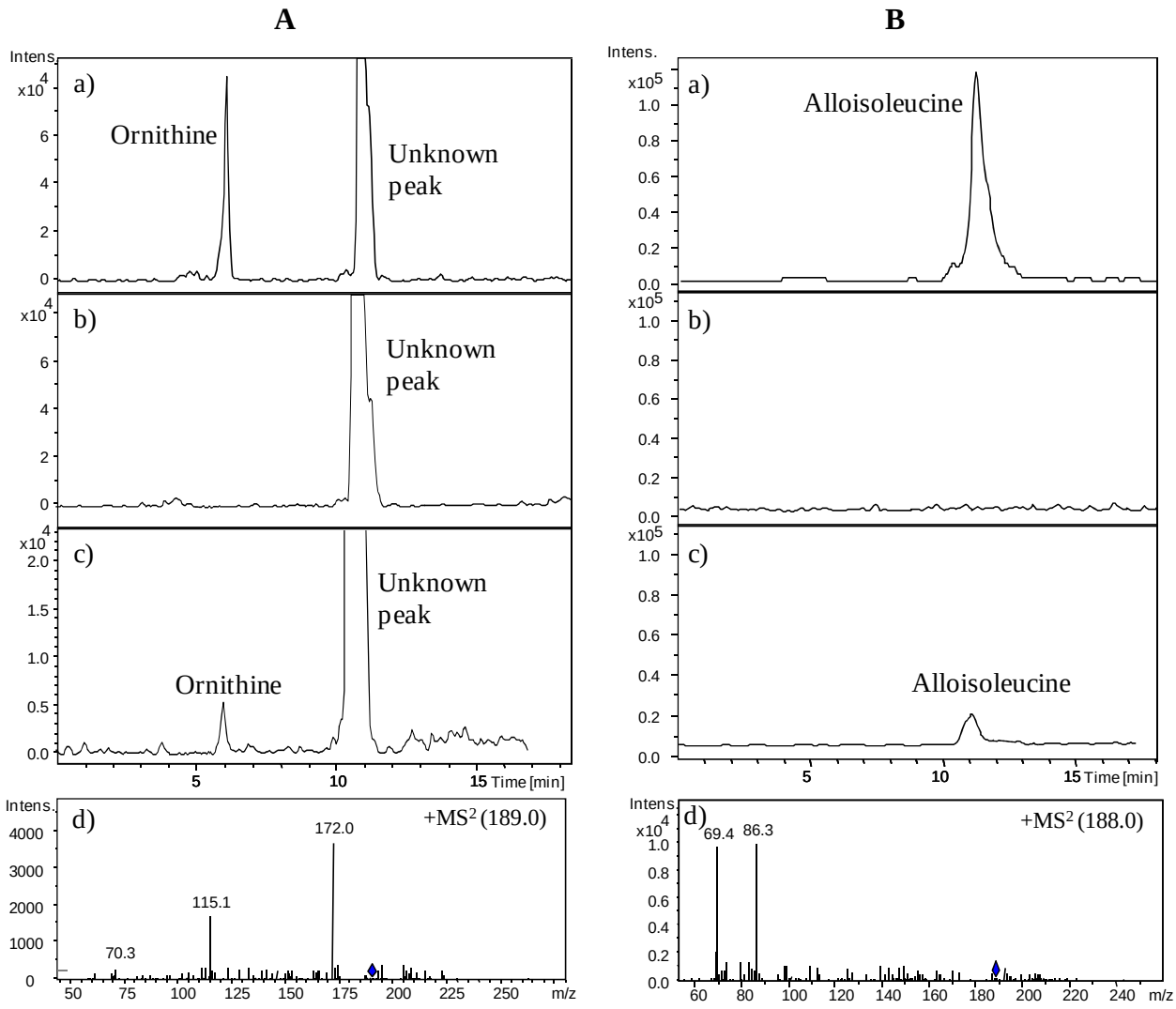
A
B
Intens.
a)
Ornithine
Unknown peak
b)
Unknown peak
c)
Unknown peak
Ornithine
Time [min]
d)
+MS2 (189.0)
172.0
115.1
70.3
m/z
Alloisoleucine
Alloisoleucine
+MS2 (188.0)
69.4
86.3

Table 1. Botanical origin, number of samples and name for the vegetable oils samples used in this work.

Origin	Number of samples	Name
Sunflower oil	3	RSO-1 RSO-2 RSO-3
Corn oil	3	RCO-1 RCO-2 RCO-3
Soybean oil	3	RSYO-1 RSYO-2 RSYO-3
Hojiblanca extra virgin olive oil	3	HEVOO-1 HEVOO-2 HEVOO-3
Arbequina extra virgin olive oil	3	AEVOO-1 AEVOO-2 AEVOO-3
Picual extra virgin olive oil	3	PEVOO-1 PEVOO-2 PEVOO-3

Table 2. Optimization of the reaction temperature and time for the derivatization procedure.

		% Derivatization reaction[a)]	
		Phenylalanine	Tryptophan
Temperature of reaction [b)]	60 ºC	95.1	95.4
	70 ºC	97.4	97.6
	80 ºC	98.9	98.9
	100 ºC	99.0	99.0
Time of reaction[c)]	30 min	100.0	100.0
	20 min	98.9	98.9
	10 min	85.4	86.8

[a)] % Reaction = 100 x $Ac_{derivatized\ compound}/(Ac_{derivatized\ compound} + Ac_{underivatized\ compound})$; Ac, corrected area

[b)] Time of reaction, 20 minutes.

[c)] Temperature of reaction, 80 ºC in order to avoid the butanol boiling point (117.73 ºC).

Table 3. Analytical characteristics of the CE-MS^2 developed method[a)].

Compound	LOD (ng/g)	LOQ (ng/g)	Linearity[b)] R^2	Linearity[b)] Intercept	Precision (Ac, RSD(%))[c)] Instrumental Repeatability[d)]	Precision (Ac, RSD(%))[c)] Method Repeatability[e)]	Precision (Ac, RSD(%))[c)] Intermediate Precision[f)]	Recovery[g)] Low level	Recovery[g)] High level
Ornithine	0.04	0.06	0.997	0.4 (±3.4) x 10^4	2.2	1.9	2.4	90±2	84±1
β-Alanine	0.04	0.06	0.990	0.6 (±2.3) x 10^4	2.0	1.8	5.1	87±1	91±1
GABA	0.04	0.06	0.995	4.8 (±5.7) x 10^4	2.9	4.3	5.4	88±3	86±2
Alloisoleucine	0.04	0.06	0.994	2.1 (±2.3) x 10^4	2.8	4.7	6.3	83±1	87±1
Citrulline	0.19	0.31	0.995	1.3 (±1.9) x 10^4	-	-	-	84±3	83±4
Pyroglutamic acid	0.19	0.31	0.990	3.9 (±5.1) x 10^4	2.9	4.6	6.9	91±2	89±1

[a)] Experimental conditions as in Fig. 3.
[b)] Six standard solutions at different concentration levels (LOQ-100LOQ) injected in triplicate during three days. Values in parentheses are confidence intervals at 95%: $\pm t \times s_{intercept}$.
[c)] Ac means corrected peak area (peak area divided by time).
[d)] Obtained from six consecutive injections of RSO-1in the same day.
[e)] Obtained from three individual RSO-1 samples injected by triplicate in the same day.
[f)] Assessed from three individual RSO-1 samples injected in triplicate in three consecutive days.
[g)] Recovery for HEVOO-1 samples spiked at low level (5 ng of each compound) and at high level (0.5 µg of each compound). Average ± standard deviation (n=3).

Table 4. Botanical origin, name of the vegetable oils samples used in this work and the quantitation of the non-protein amino acids in the samples (n=3). ND: not detected (< LOD).

		Quantitation (ng/g)					
Origin	**Sample Name**	**Ornithine**	**β-Alanine**	**GABA**	**Alloisoleucine**	**Citrulline**	**Pyroglutamic acid**
Sunflower oil	RSO-1	2.7 ± 0.3	1.4 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	ND	5.7 ± 0.8
	RSO-2	0.8 ± 0.7	1.0 ± 0.1	0.4 ± 0.1	1.2 ± 0.4		4.4 ± 0.5
	RSO-3	1.0 ± 0.1	2.8 ± 0.4	0.7 ± 0.2	2.1 ± 0.1		7.2 ± 0.7
Corn oil	RCO-1	1.2 ± 0.2	1.1 ± 0.2	0.5 ± 0.2	5.4 ± 0.7	ND	5.6 ± 0.4
	RCO-2	0.9 ± 0.3	2.5 ± 0.2	0.7 ± 0.2	2.8 ± 0.1		7.6 ± 0.9
	RCO-3	1.3 ± 0.3	1.5 ± 0.1	0.6 ± 0.1	1.4 ± 0.5		5.6 ± 0.8
Soybean oil	RSYO-1	2.0 ± 0.6	2.0 ± 0.2	0.37 ± 0.01	0.6 ± 0.1	ND	19 ± 1
	RSYO-2	0.8 ± 0.1	1.8 ± 0.2	0.62 ± 0.01	1.2 ± 0.1		10 ± 1
	RSYO-3	1.0 ± 0.1	1.8 ± 0.3	0.47 ± 0.02	0.7 ± 0.1		13 ± 1
Hojiblanca extra virgin olive oil	HEVOO-1	ND	0.23 ± 0.01	0.12 ± 0.01	ND	ND	0.59 ± 0.01
	HEVOO-2		0.25 ± 0.01	0.17 ± 0.04			0.75 ± 0.09
	HEVOO-3		0.26 ± 0.04	0.10 ± 0.01			ND
Arbequina extra virgin olive oil	AEVOO-1	ND	0.33 ± 0.01	0.09 ± 0.02	ND	ND	0.91 ± 0.02
	AEVOO-2		0.22 ± 0.01	0.06 ± 0.01			0.87 ± 0.01
	AEVOO-3		0.36 ± 0.05	0.14 ± 0.02			ND
Picual extra virgin olive oil	PEVOO-1	ND	0.23 ± 0.01	0.08 ± 0.04	ND	ND	0.41 ± 0.02
	PEVOO-2		0.12 ± 0.02	< LOQ			ND
	PEVOO-3		ND	ND			ND
Mixtures of HEVOO-1 with RSYO-3	10%	0.22 ± 0.03	0.48 ± 0.03	0.22 ± 0.01	0.12 ± 0.02	ND	2.27 ± 0.04
	5%	0.10 ± 0.01	0.31 ± 0.04	0.19 ± 0.05	0.09 ± 0.01		1.14 ± 0.02
	2%	< LOQ	0.28 ± 0.03	0.12 ± 0.03	0.07 ± 0.01		0.65 ± 0.01